Parasitol Res (2016) 115:2957–2965
DOI 10.1007/s00436-016-5049-7

ORIGINAL PAPER

Serodiagnosis of fasciolosis by fast protein liquid chromatography-fractionated excretory/secretory antigens

Kobra Mokhtarian[1] · Lame Akhlaghi[1] · Ahmad Reza Meamar[1] · Elham Razmjou[1] · Kourosh Manouchehri Naeini[2] · Samaneh Gholami[3] · Masoomeh Najafi Samei[3,4] · Reza Falak[3,4]

Received: 14 March 2016 / Accepted: 7 April 2016 / Published online: 30 April 2016

Abstract In several studies, different antigenic preparations and diverse immunological tests were applied for serodiagnosis of *Fasciola hepatica* infections. Most of these preparations showed cross-reactivity with proteins of other parasites. Application of purified antigens might reduce these cross-reactivities. Here, we used fast protein liquid chromatography (FPLC)-fractionated extracts of *F. hepatica* excretory/secretory antigens (E/S Ags) for serodiagnosis of human and sheep fasciolosis. To develop an improved diagnostic method, we fractionated *F. hepatica* E/S Ags by anion exchange chromatography on a Sepharose CL-6B column and then tested the serodiagnostic values of the fractions. We used sera from *F. hepatica*-infected human and sheep as positive controls. Sera from patients with hydatidosis and strongyloidiasis were used for cross-reactivity studies. Enzyme-linked immunosorbent assays (ELISA) of the second FPLC peak, containing 20, 25, and 70 kDa proteins, discriminated between *F. hepatica*-infected and uninfected human and sheep samples. Fractionation of *F. hepatica* E/S Ags by FPLC is a fast and reproducible way of obtaining antigens useful for

Highlights
- *Fasciola hepatica* antigens were fractionated by FPLC anion exchange chromatography.
- Fractions containing the 17-, 48-, and 50-kDa proteins cross-reacted with sera from hydatidosis patients.
- The 12-, 14-, and 25-kDa proteins discriminate fasciolosis with high sensitivity and specificity.
- FPLC is a fast, simple, and reproducible way to purify *F. hepatica* immunogens.
- FPLC-fractionated antigens are useful for serodiagnosis of human and sheep fasciolosis.

✉ Lame Akhlaghi
Akhlaghi.l@iums.ac.ir

✉ Reza Falak
Falak.r@IUMS.ac.ir; rezafalak@gmail.com

Kobra Mokhtarian
K.mokhtarian@yahoo.com

Ahmad Reza Meamar
ameamar@yahoo.com

Elham Razmjou
Elhamrazmjou@yahoo.com

Kourosh Manouchehri Naeini
K_manouchehrii@yahoo.com

Samaneh Gholami
s.gholamiii@yahoo.com

Masoomeh Najafi Samei
Najafi.M1352@yahoo.com

[1] Department of Parasitology and Mycology, School of Medicine, Iran University of Medical Sciences (IUMS), Tehran, Iran

[2] Department of Parasitology and Mycology, School of Medicine, Shahrekord University of Medical Science, Shahrekord, Iran

[3] Immunology Research Center, Iran University of Medical Science (IUMS), Tehran, Iran

[4] Department of Immunology, School of Medicine, Iran University of Medical Sciences (IUMS), Tehran, Iran

serodiagnosis of human and sheep fasciolosis with acceptable sensitivity and specificity.

Keywords Fasciolosis · Fast protein liquid chromatography · Fractionated excretory/secretory antigens

Introduction

Fasciolosis is a cosmopolitan parasitic disease caused by *Fasciola hepatica.* This infection occurs in a variety of mammals, including humans. It is highly prevalent in sheep due to their close contact with infective pasture. This parasite commonly inhabits the hepato-biliary system of the affected hosts (Mas-Coma et al. 2005). Annual economic loss caused by the disease in domestic animals was estimated to be US $2 billion, mainly due to condemned livers, reduced milk yields, fertility disorders, and diminished meat production (Spithill et al. 1999). During the past two decades, the disease has emerged as a serious problem in veterinary medicine and human health.

In 1999, over 10,000 human cases were reported in Gilan, the Northern Province of Iran (Rokni et al. 2002). Sporadic cases have also been reported in other provinces including Kurdistan, Zanjan, Mazandaran, Tehran, and Azarbaijan (Mohamed et al. 2004). Prevalence of the infection in domestic animals has been reported from 1.07 % to more than 50 % in different areas of Iran (Rokni et al. 2010).

Human fasciolosis is commonly diagnosed by detection of parasite eggs in stool, but this method is not reliable and has poor sensitivity in diagnosis during the acute ectopic stage. Recently, immunodiagnostic methods, including enzyme-linked immunosorbent assays (ELISA), have been widely applied in parasitic diagnoses (Hillyer 1999). In several studies, different antigenic preparations and diverse immunological tests were used for serodiagnosis of *F. hepatica* infections. The excretory/secretory antigens (E/S Ags), as well as some somatic and metabolic products (enzymes, fatty acid-binding proteins, etc.) of this parasite, exhibit immunogenic properties and could trigger humoral immune responses. Unfortunately, most of them cross-react with antigens of other parasites. We proposed that application of purified antigens might reduce this cross-reactivity. In this study, fast protein liquid chromatography (FPLC)-fractionated extracts of *F. hepatica* E/S Ags were applied for serodiagnosis of fasciolosis and their specificity and sensitivity were evaluated.

Materials and methods

Parasite and serum samples

Livers from *F. hepatica*-infected sheep were collected from the Kahrizak slaughterhouse located in Tehran, Iran. Adult live flukes were removed from the infected livers, washed six times with warm sterile phosphate-buffered saline (PBS), and used to prepare total extract and E/S Ags.

Sera from 14 fasciolosis human patients, whose infections were confirmed in a medical laboratory using a commercial ELISA kit (Pishtaz Teb, Iran) and routine stool exams, and from 20 healthy people who had no evidence of intestinal parasite infections and negative stool exams were used as positive and negative human samples, respectively.

In addition, sera from 12 infected sheep, in which fasciolosis was obvious during examination of the liver at the slaughterhouse, and from 13 healthy animals were applied as positive and negative animal samples for the development of the immunoassays. To determine the cross-reactivity of the antibodies from different parasite infections, we applied four strongyloidiasis and four hydatidosis patients' sera in the study. Moreover, to assess the cross-reactivity of infected sheep sera with other parasitic diseases, we used sera from sheep infected with *Echinococcus granulosus*, *Dicrocoelium dendriticum*, and *Taenia hydatigena* in the experiments.

Preparation of *F. hepatica* antigens

Preparation of F. hepatica E/S antigens

After washing the flukes with PBS, the parasites were incubated in RPMI-1640 medium (1 fluke per 2 ml of medium; Gibco-BRL, Gaithersburg, MD, USA), containing 100 IU/ml of penicillin, 100 μg/ml of streptomycin, and 1X anti-protease cocktail at 37 °C for 24 h in a 5 % CO_2 incubator as described elsewhere (Spithill et al. 1999). The supernatant, containing the E/S product of the parasites, was collected and centrifuged at 10,000×*g* for 30 min at 4 °C. Afterwards, the clear supernatant was concentrated via ultrafiltration using 4 kDa membrane filters (Eppendorf, Germany). The protein concentration of the supernatant was estimated using the Bradford method with bovine serum albumin (BSA) as the standard, and the product was stored at −20 °C.

Preparation of F. hepatica somatic antigens

Adult flukes were washed six times in PBS, pH 7.3, to remove debris. One gram of the flukes was added to 3 ml of lysis buffer (50 mM Tris pH 8.0 containing 2 mM EDTA, 2 mM 2-ME, 100 mM NaCl, 5 % glycerol, and 1X anti-protease cocktail) and homogenized with an electrical homogenizer (IKA®T25 digital ULTRA-TURRAX, Germany). The homogenate was centrifuged at 13,000×*g* for 30 min. The supernatant containing soluble products was removed and stored at −20 °C.

SDS-PAGE of *F. hepatica* proteins

F. hepatica E/S and somatic antigens were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Briefly, 10 μg of each extract were loaded onto a 4 % stacking and 15 % separating gel. After electrophoresis, the protein bands were visualized with Coomassie blue G-250 staining and their apparent molecular weights were determined by comparing with wide molecular weight protein marker (Sinaclon, Tehran, Iran).

FPLC fractionation and silver nitrate staining of the E/S antigen

The E/S antigen was fractionated by anion exchange chromatography using an AKTA PRIME Plus FPLC system (GE Healthcare Biosciences, Uppsala, Sweden). The concentrated E/S Ags were dialyzed against 20 mM Tris–HCl pH 8.0. Then, 3 mg of the E/S Ags was applied onto a DEAE-Sepharose CL-6B resin column and eluted with 20 mM Tris–HCl pH 8.0 containing 1 M NaCl. The sample was eluted at a flow rate of 1 ml/min. Elution was monitored by measuring at 280 nm. Similar peaks from several runs were pooled and concentrated by lyophilization. The lyophilized fractions were resuspended in 1 ml of distilled water and separated by 15 % SDS-PAGE. Proteins were stained with silver nitrate, and their apparent molecular weights were compared with the E/S Ag pattern.

Western blotting

We studied the immunoreactivity of *F. hepatica* E/S Ags and the contents of the fractions by Western blotting. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, USA) for 90 min at 0.7 mA/cm^2 using a semi-dry transfer apparatus (PeQlab). After the transferring step, the membrane was weakly stained for about 3–5 min with 0.2 % Ponceau S (Sigma-Aldrich, UK). Then, the membrane was dried and cut into 2-mm-wide stripes. After destaining with distilled water, the strips were washed and blocked with 2 % BSA overnight at 4 °C and then 1:100 diluted human or 1:500 diluted ovine sera were added at room temperature (RT) for 2 h on a rocker. The strips were then washed three times with PBS containing 0.05 % Tween 20 (PBS-T) for 10 min and incubated either with horseradish peroxidase (HRP)-conjugated polyclonal anti-human antibody (Sigma-Aldrich, Germany) diluted 1:1000 in BSA for 60 min at RT or HRP-conjugated polyclonal rabbit anti-sheep antibody (Sina Biotech, Iran) diluted 1:5000 and incubated as above. The strips were washed and incubated with chemiluminescent substrate (Luminol and H_2O_2) for 2 min at RT. Finally, the reactive bands were detected on X-ray film within 10–20 s under safelight conditions as previously described (Falak et al. 2013).

ELISA

The IgG reactivity of total E/S Ags and FPLC fractions were determined by an indirect ELISA as described by Morales et al. with some modifications (Morales and Espino 2012). To identify the optimal dilutions of antigen and antibody in ELISA, a checker board titration assay was carried out prior to the final experiments. Briefly, wells of Polysorb micro titration plates (Nunk, USA) received 50 μl of 2 μg/well E/S Ag or 2 μg/well of fractionated proteins in 0.1 M bicarbonate buffer pH 9.6 and incubated overnight at 4 °C. After washing with PBS-T, wells were blocked with 250 μl of 2 % BSA/well and the plates were incubated for 90 min at 37 °C. Following a brief washing, 50 μl of 1:100 diluted patients' or 1:500 diluted ovine sera were added and the plates were incubated for 2 h at 37 °C. Plates were then washed six times with PBS-T and received 50 μl/well of 1:1000 diluted HRP-conjugated polyclonal anti-human IgG or 1:5000 diluted HRP-conjugated polyclonal rabbit anti-sheep antibody, respectively, and incubated for 60 min at 37 °C. The plates were then washed four times, and 50 μl of tetramethylbenzidine (TMB)/H_2O_2 mixture was added as the chromogen/substrate solution. Following 15 min incubation in the dark at RT, the enzymatic reaction was stopped by adding 50 μl of 2 N sulfuric acid, and the optical density (OD) was measured at 450 nm using an ELISA micro-plate reader (BioHit BP 800, Finland).

Mass spectrometry and database search

Following anion exchange chromatography, the purified fractions were subjected to SDS-PAGE for second purification step based on molecular weights and the resolved protein bands were visualized by colloidal Coomassie blue staining. After mild destaining in distilled water, the immunoreactive bands were manually excised from the gel and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) at Yurk Company, England. Mass spectrometry was performed using an ABI 4800 MALDI-TOF/TOF analyzer (Applied Biosystems, Foster City, CA, USA). Mass spectra were recorded in reflector-positive mode with a scanning range of 900–4000 Da. Five monoisotopic precursors from each purified fraction with signal/noise (*S*/*N*) ratios greater than 200 were selected for MS/MS analysis. The Swiss-Prot and NCBI databases for the peptide mass maps were searched using the Mascot search engine (Matrix-Science, London, UK). Searches were done with tryptic specificity allowing one missed cleavage and a tolerance on the mass measurement of 100 ppm in MS mode and 0.5 Da for MS-MS ions. Oxidation and carbamidomethylation of the cysteines were also selected as the fixed possible

modification of fragmented peptides. A protein identification was considered accurate when at least three peptides were identified with an overall Mascot score greater than 60.

Data analysis

Statistical analyses were performed using the Student's *t* or Mann–Whitney tests using SPSS version 20. The ELISAs were performed in duplicate, and the results were expressed as the mean absorbance value (*A*450) for each determination. The mean *A*450 plus 3 standard deviations (SD) of the negative control group was used as the cutoff limit between positivity and negativity status for fasciolosis. The diagnostic sensitivity and specificity were calculated by common statistical methods as described elsewhere (Morales and Espino 2012).

Results

SDS-PAGE

The somatic and E/S products of *F. hepatica* were separated by SDS-PAGE under reducing conditions. The electrophoretic patterns of the extracts were similar, with protein bands ranging from 4 to 100 kDa (Fig. 1); however, the intensities of the bands differed. The somatic extract contained proteins with apparent molecular weights of 12–14, 25, 33–39, and 48–70 kDa, while the more intense bands of the E/S product were located at 20–27 and 40–48 kDa. Less intense bands were observed at 15–20, 65, and 75–100 kDa.

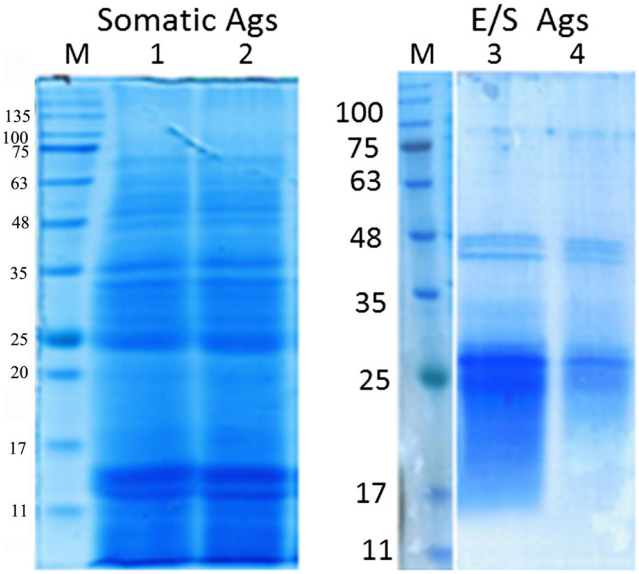

Fig. 1 SDS-PAGE of *F. hepatica* somatic and excretory-secretory antigens. *F. hepatica* somatic (*lanes 1 and 2*) and E/S Ags (*lanes 3 and 4*) were separated on 15 % polyacrylamide gels and stained with brilliant Coomassie blue G-250. *MW* protein molecular weight marker (Sinaclon, Iran)

Analysis of the FPLC fractions

Two major protein peaks were observed following anion exchange chromatography of *F. hepatica* E/S Ags (Fig. 2). The fractions corresponding to these peaks were collected and referred to as P1 and P2. The contents of both peaks were lyophilized and subjected to SDS-PAGE and silver nitrate staining (Fig. 3a). P1 and P2 eluted at 65 and 75 % NaCl concentrations, respectively.

On Western blots, sera from fasciolosis patients reacted with proteins of 12, 19, 48, and 70 kDa in the 20th elute (P1) and, 20, 25, 63, and 70 kDa in the 24th elute (P2). The strongest immunoreactivity was seen with the 25-, 63-, and 75-kDa proteins, while faint bands were seen at 12 and 14 kDa (Fig. 3b). Similar results were seen with ovine sera, with bands at 14, 17, and 48 kDa from P1 and 18 and 20 kDa from P2. Overall, the major immunogenic protein bands were observed at approximately 12–14, 20, 25, 35, 40, 48, and 75 kDa (Fig. 3c).

Immunoblotting of E/S Ags with human and sheep sera

Sera from both humans and sheep with chronic fasciolosis reacted with proteins of 12, 14, 20, 25, 27, 35, 40, and 70 kDa (Fig. 4). The highest prevalence of immunoreactivity of human and sheep sera was with proteins of 11–15 kDa (41.17 %), 35 kDa (35.2 %), 25 kDa (23.5 %), and 27 kDa (29.4 %) (Table 1).

Pooled sera of strongyloidiasis and hydatidosis patients were used to assess cross-reactivity. Strongyloidiasis patients' sera reacted with some *F. hepatica* proteins, including the 63-kDa protein, while hydatidosis patients' sera reacted strongly with *F. hepatica* 17, 48, and 50 kDa proteins.

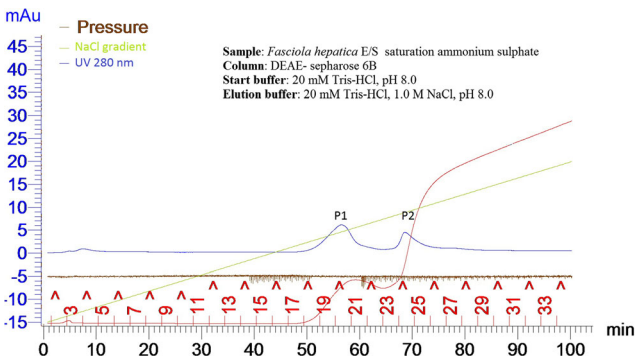

Fig. 2 Anion exchange chromatography of *F. hepatica* E/S Ags. FPLC chromatogram of *F. hepatica* E/S Ags obtained by anion exchange chromatography on a DEAE-Sepharose CL-6B column. Two typical peaks (*P1*, *P2*) were obtained when the NaCl in the elution buffer exceeded 60–70 %

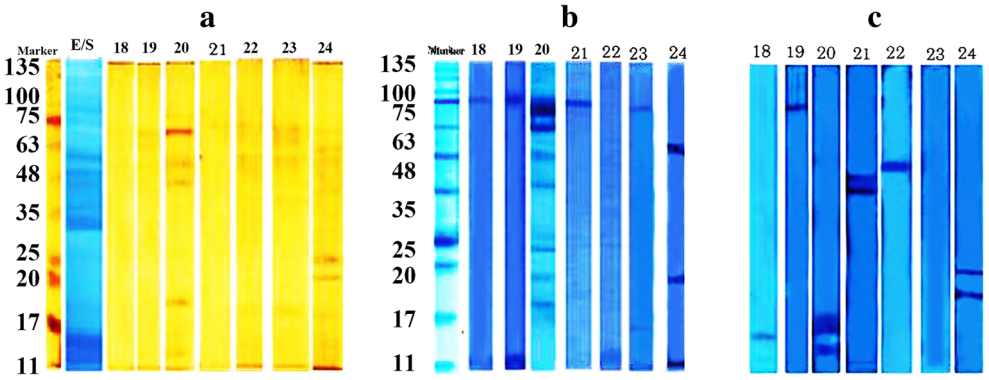

Fig. 3 SDS-PAGE of FPLC-purified fractions of *F. hepatica* E/S Ags. **a** Fractions 18–24 were electrophoresed on 15 % polyacrylamide gels and visualized by silver nitrate staining. The lane numbers 20 and 24 contain the main protein bands and correspond to peaks 1 and 2, respectively. **b**, **c** Immunoblotting of fractionated proteins with infected human and sheep sera, respectively

To focus on the specific immunogens of *F. hepatica*, we omitted the cross-reactive antigens from the study. The proteins that reacted only with infected human and ovine sera were examined to identify possible protein families and amino acid sequences.

Mass spectrometry and database search

The main immunoreactive bands were excised from the gel and identified by mass spectrometry. The MALDI-TOF analysis identified aldolase, glutathione *S*-transferase (GST), fatty acid-binding protein (FABP), enolase, and cathepsin L1 (CL1) as the major seroreactive components of the *F. hepatica* E/S Ags in human and sheep chronic fasciolosis (Table 2).

Detection of the immunoreactivity of *F. hepatica* E/S Ags by human sera

The mean OD ± 3SD values achieved for E/S Ags from positive and negative control groups were 1.48 ± 0.39 and 0.27 ± 0.05 (Fig. 5). The ODs of the positive controls were significantly higher than those of the negative controls ($P < 0.0001$). Mean ODs of strongyloidiasis and hydatidosis sera were 0.71 ± 0.31 and 0.72 ± 0.39, respectively.

When the wells were coated with P1, the mean ODs for positive controls were 1.2 ± 0.42 (Fig. 6). The ODs of strongyloidiasis and hydatidosis sera with this fraction were 0.68 ± 0.3 and 0.9 ± 0.42, respectively.

When the wells were coated with P2, the mean ODs for positive controls were 0.9 ± 0.3. However, this fraction did not strongly cross-react with strongyloidiasis or hydatidosis sera (Fig. 7).

Immunoreactivity of sheep sera with *F. hepatica* E/S Ags

Evaluation of the immunoreactivity of *F. hepatica* total E/S Ag with ovine sera showed a significant difference between mean ODs of the infected animals (1.93 ± 0.13) and the healthy controls (0.5 ± 0.11). When wells were

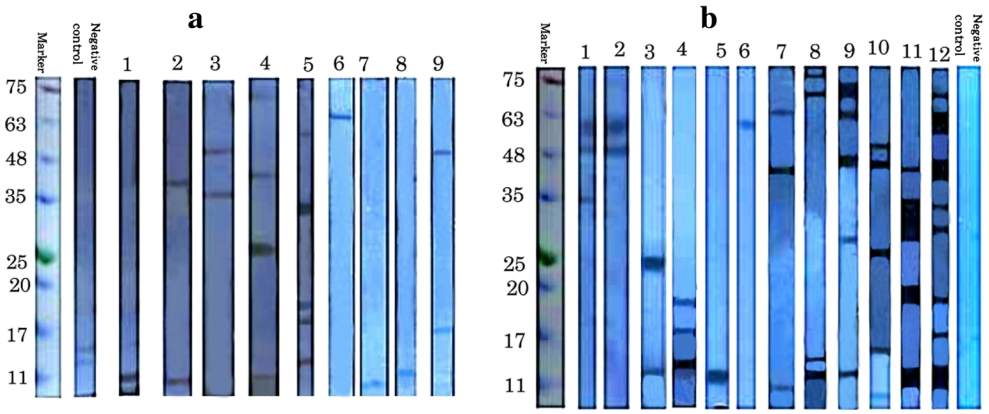

Fig. 4 Immunoblotting of *F. hepatica* E/S Ags with human and ovine sera. **a** From *left to right*, the strips refer to protein molecular weight marker. Immunoblotting of E/S Ags with sera from negative controls (pooled sera): *1–5* fasciolosis patients, *6–7* strongyloidiasis patients, and *8–9* hydatidosis patients, respectively. **b** From *left to right*, the strips refer to protein molecular weight marker: *1–12* immunoblotting with sera from *F. hepatica*-infected ovine and pooled sera from negative controls, respectively

Table 1 Immunoreactive protein bands found via immunoblotting of *Fasciola hepatica* antigens with human and sheep sera

Sample ID	Immunogenic proteins of *F. hepatica* to human sera	Immunogenic proteins of *F. hepatica* to sheep sera
1	12	35, 48
2	12	48, 60
3	35, 48, 65–70	14, 25
4	27,40	11–15, 17, 19
5	14, 17, 19, 25–27, 35	11–14
6	63	60
7	–	40, 63
8	–	11–15, 30, 35, 65, 75
9	17, 48, 50	14, 27, 40–48, 63, 70–75
10	–	15, 25–27, 40, 48
11	–	11–15, 17–20, 25, 27–35, 40
12	–	11–15, 17, 19, 27, 30, 35–40, 50–63

coated with E/S Ags, P1, and P2, the mean ODs with infected sheep sera were 1.93 ± 0.13, 1.80 ± 0.137, and 1.61 ± 0.21, respectively, while ODs with healthy sheep sera were 0.42 ± 0.07, 0.36 ± 0.04, and 0.26 ± 0.04, respectively. The mean OD values with total E/S Ags, P1, and P2 using sheep sera with other similar parasitic diseases were 0.66, 0.47, and 0.34, respectively. Overall, these results, similar to results obtained with human sera, showed that there is significant difference between mean OD of the patients sera with of 24th fraction compared to healthy controls, while the total E/S Ags and the P1 fraction showed a higher OD with endemic parasites.

Discussion

We found that the somatic and E/S products of *F. hepatica* share several identical molecules, including the 15–25- and 48-kDa proteins. These results agree with the reports of Meshgi et al. (2008) and Upadhyay and Kumar (2002). Based on blotting results, we found that the specific immunogenic protein bands of the E/S product were 20–27 and 40–48 kDa molecules. Moreover, 12–14, 25, 33–39, and 48–70 kDa proteins comprised the main somatic immunogens of *F. hepatica*. Allam et al. (2002) showed that some low molecular weight proteins of the whole parasite extract, ranging from 25 to 48 kDa, comprise the main antigenic molecules

Table 2 Mass spectrometry results for fractionated proteins of *Fasciola hepatica*

Protein fraction	Protein name/species	Nominal mass (M_r)/PI	Protein score	Accession key	Observed mass	Peptide sequence
Unknown 12 kDa immunogen	Fatty acid-binding protein type 2	14,926/5.93	187	gi|124012088	1681.8058	^{35}RNEKPEFTFELEGNKM^{48}
	Fatty acid-binding protein Fh15	14,760/5.91	172	gi|47115698	2184.9514	^{61}KTTFTFTGEEFKDETFDNRT^{78}
					1441.6479	^{10}KYGHSENMEAYLKK^{21}
					1821.9578	^{33}KILNAKPEFTFTLEGNKM^{48}
Unknown 14 kDa immunogen	Hemoglobin F2	16,681/7.03	75	gi|159461074	1307.7157	^{108}KDQFTGAAPIFIKF^{119}
	Fatty acid-binding protein type 3	14,671/9.02	72	gi|47116941	2264.0508	^{59}KTTVISFTFGEEFKEETADGRT^{78}
Unknown 25 kDa immunogen	Chain A, *Fasciola hepatica* Sigma Class Gst	24,690/8.86	116	gi|292659520	1778.8253	^{84}KMMGETDEEYYLIERI^{97}
	Mu-glutathione transferase, partial [*Fasciola hepatica*]	24,592/5.88	66	gi|159058	1306.6948	^{84}RISMIEGAAMDLRI^{95}
Unknown 36 kDa immunogen	Cathepsin L-like [*Fasciola hepatica*]	35,611/5.79	345	gi|137740802	2321.9717	^{170}KQFGLETESSYPYTAVEGQCRY^{189}
					1768.8569	^{199}KVTGYYTVHSGSEVELKN^{214}
					1191.5632	^{273}KNSWGLSWGERG^{282}
Unknown 39 kDa immunogen	Fructose-bisphosphate aldolase class I, partial [*Clonorchis sinensis*]	98,805/9.66	86	gi|358253990	2357.0938	^{622}kGVVPLAGSLNECTTQGLDG LAERC^{644}

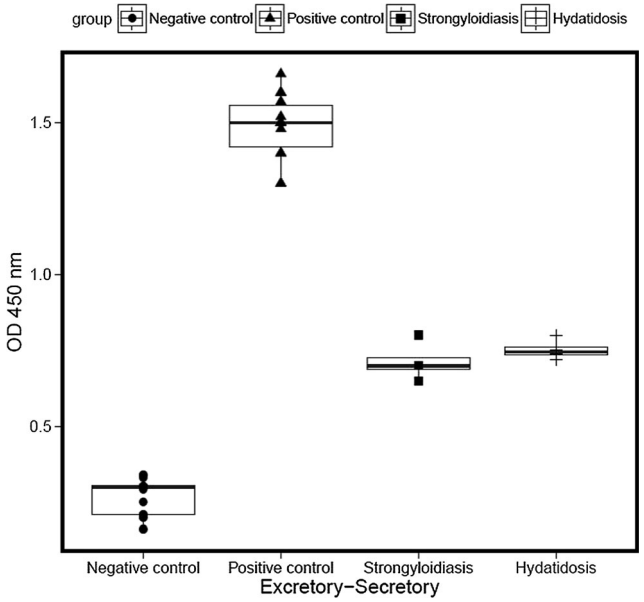

Fig. 5 Comparison of the immunoreactivity *F. hepatica E/S* Ags with human sera by ELISA. Immunoreactivity of fasciolosis, strongyloidiasis, and hydatidosis patients' sera compared to healthy controls using *F. hepatica* E/S Ag-coated ELISA plates. The *dashed line* represents the cutoff point between seronegative and seropositive populations, which was calculated as the mean plus 3 standard deviations of the healthy population (mean + 3SD)

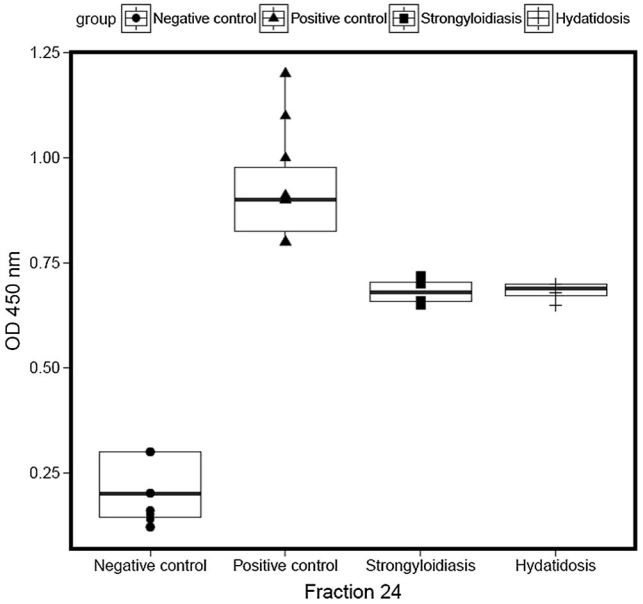

Fig. 7 Comparison of the immunoreactivity of *F. hepatica* 24th fraction with different groups of human sera by ELISA. Analysis of sera obtained from healthy controls, strongyloidiasis, and hydatidosis patients by ELISA using fraction 24 (*second peak*) of anion exchange chromatography as the coating antigen. *Dashed line* represents the cutoff point between seronegative and seropositive populations, which was calculated as 3 standard deviations from the mean result of the seronegative population (mean + 3SD)

of *F. hepatica.* These differences could be due to different parasite isolates from different host species, geographic variations, or the different methods of protein extraction. E/S Ags of *F. hepatica* contain several glycoproteins, including hemoglobin and cysteine proteases, which are usually recognized as immunogens by the host immune system (McGonigle and Dalton 1995).

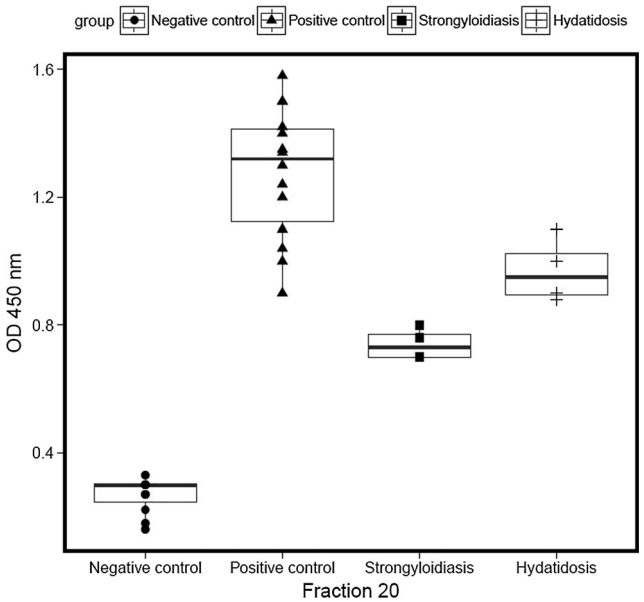

Fig. 6 Comparison of the immunoreactivity of *F. hepatica* fraction 20 with human sera by ELISA. Optical densities of ELISAs using sera from healthy controls, strongyloidiasis, and hydatidosis patients on fraction 20 (*first peak*) of anion exchange chromatography as the coating antigen. The *dashed line* represents the cutoff point between seronegative and seropositive populations, which was calculated as the mean plus 3 standard deviations of the seronegative population (mean + 3SD)

Based on potential antigenicity of the E/S product, its purified antigens were used in several immunoassays for serodiagnosis of animal and human fasciolosis (Carnevale et al. 2001; Cornelissen et al. 2001). We used the E/S product and its purified proteins as the antigen source. Antigenic proteins with apparent molecular weights of 12, 14, 19, 20, 25, 27, 35, 40, and 70 kDa reacted with both human and sheep sera. None of these proteins cross-reacted with *Strongyloides stercoralis*-infected patients' sera, which has the same geographical prevalence as fasciolosis and hydatidosis, which are endemic zoonotic parasite diseases in Iran. Our results agree with those of Rokni et al. (2004), who found that the 14-kDa E/S protein and the 27- and 29-kDa somatic extract proteins are major immunogenic molecules. However, Gonenc et al. found that the 33-, 39.5-, and 42-kDa proteins are specific E/S Ag immunogens. In addition, they reported a 63-kDa protein as a shared antigen present in both the somatic and E/S products (Gonenc et al. 2004).

Our study showed that the 63-kDa protein is a major cross-reactive protein in strongyloidiasis, while the 17-, 48-, and 50-kDa proteins cross-reacted with hydatidosis patients' sera. This is likely due to shared epitopes between

E/S Ags of *F. hepatica* and *S. stercoralis* and *E. granulosus* (Mohamed et al. 2004). Yamano et al. showed that the Gal-beta1-6Gal sequence was a common carbohydrate epitope between *Echinococcus multilocularis* and *E. granulosus*. Cross-reactivity of this sequence with *F. hepatica* has been reported in several studies. Furthermore, it was demonstrated that Gal-beta1-6Gal is a common epitope of various other parasites (Yamano et al. 2009).

We did not assay acute fasciolosis serum samples in this study, but in other studies, 25–30 kDa proteins of E/S Ags showed specificity in both acute and chronic phases of fasciolosis in rabbits, cows, and sheep (Rivera-Marrero et al. 1988). Sampaio-Silva et al. reported that 25–27 kDa components of E/S products of adult flukes could recognized by fasciolosis patients' sera (Sampaio-Silva et al. 1996).

Our findings showed that the total E/S product is not a specific preparation for serodiagnosis of human and sheep fasciolosis by ELISA. This finding agrees with previous studies in cattle (Cornelissen et al. 1999; Bossaert et al. 2000), other animals, and humans (Mezo et al. 2003; Demerdash et al. 2011). Similar to Western blotting experiments of Adelaida et al., we observed no reactive protein bands in the negative controls (Valero et al. 2012).

Fractionation of the E/S product by ion exchange chromatography enabled us to partially purify the 17-, 20-, 25, and 70-kDa immunogenic proteins by sodium chloride gradient. The purified proteins did not show cross-reactivity with endemic parasites.

Overall, FPLC fractions revealed more specific results for serodiagnosis of human fasciolosis than those obtained from somatic extracts or total E/S products. This finding is also in line with previous reports (Ashrafi et al. 2006; Rokni et al. 2010; Sarkari et al. 2012; Valero et al. 2012). Coating of the ELISA plates with the second FPLC peak containing the 17-, 20-, 25-, and 70-kDa proteins revealed 100 % sensitivity and specificity, similar to findings of Mezo et al., who also used FPLC for protein fractionation (Mezo et al. 2003). In the present study, the 27-kDa protein was recognized by human and sheep sera. The lower molecular weight antigens were not found in other parasites. It was predicted that CL1 could play a vital role in the adaptive immune response to *F. hepatica* infection (Mezo et al. 2003). Later, Farghaly et al. reported that the 27-kDa protein of the E/S product is 100 % sensitive and specific for diagnosis of fasciolosis and could be nominated as the most immune reactive protein (Farghaly et al. 2009). This finding was determined by Falcon assay screening test (FAST-ELISA) and immunoblotting. However, we found protein bands with apparent molecular weights of 11–15, 25, 27, and approximately 35 kDa had prominent immunoreactivity in ovine and human fasciolosis, similar to other studies (Mezo et al. 2003; Hacariz et al. 2011; Morales and Espino 2012). Fractionation of the E/S contents via chromatography helped us further resolve the components by SDS-PAGE and subsequent MS-MS analysis.

Our study identified GST, FABP, CL1, and aldolase as the major immunoreactive components of the *F. hepatica* E/S products. Notably, these proteins were recently found to be highly abundant either on the surface or in internal organs of *F. hepatica* (Robinson et al. 2009; Hacariz et al. 2011; Morales and Espino 2012). Similar to Espino et al., in this study, different isoforms of *F. hepatica* FABP (type 2 and type 3) were purified (Espino and Hillyer 2001). The abundance of FABP on the surface of *F. hepatica* indicates that this protein plays role as an antioxidant as well as a transporter of fatty acids from the environment.

The purified 25 kDa protein was identified as GST, an enzyme found in all animals with a confirmed role in detoxification and removal of harmful molecules. GST is permanently expressed on tegumental surfaces of *F. hepatica* and is also excreted by the parasite. The immunogenicity of GST was also previously demonstrated (Abath and Werkhauser 1996).

Aldolase (39 kDa), an essential glycolytic enzyme in carbohydrate metabolism, is vital for energy production, activity, and parasite survival (Espino and Hillyer 2001).

In summary, we demonstrated that *F. hepatica* fractions are excellent antigens for serodiagnosis of human and sheep fasciolosis. Western blotting showed that bands with MW of 11–15, 25–27, and 39 kDa are the most prominent and specific immunoreactive components of *F. hepatica* E/S Ags representing FABP, GST, CL1, and aldolase, respectively. Based on these results, it seems that combination of ELISA and Western blotting with *F. hepatica* fractions could be applied for specific and sensitive diagnoses of human and sheep fasciolosis. This study was carried out based on the analysis of human and ovine fasciolosis sera, and it seems that the antigens in the second peak of anion exchange chromatography, containing the 20-, 25-, and 70-kDa proteins, will be useful for serodiagnosis of fasciolosis without cross-reactivity with other parasite infections such as hydatidosis.

Conclusion

Fractionation of *F. hepatica* ES-Ag by this procedure is a fast, simple, and reproducible method for serodiagnosis of human and sheep fasciolosis. The production of the recombinant forms of these immunogenic proteins could facilitate the development of more sensitive and specific detection methods.

Acknowledgments This study was supported by grant 24488 from the Iran University of Medical Sciences (IUMS), Tehran, Iran. We thank Professor Eshrat beigom Kia from the School of Public Health, Tehran

University of Medical Sciences (Tehran, Iran) for providing strongyloidiasis sera and Professor AbdolFatah Sarafinejhad from the Noor Referral Laboratory (Tehran, Iran) for providing us with fasciolosis patients' sera.

References

Abath FG, Werkhauser RC (1996) The tegument of Schistosoma mansoni: functional and immunological features. Parasite Immunol 18:15–20

Allam AF, El-Agamy E-SI, Helmy MH (2002) Molecular and immunological characterization of *Fasciola* species. Br J Biomed Sci 59: 191–195

Ashrafi K, Valero MA, Panova M, Periago MV, Massoud J, Mas-Coma S (2006) Phenotypic analysis of adults of *Fasciola hepatica*, *Fasciola gigantica* and intermediate forms from the endemic region of Gilan, Iran. Parasitol Int 55(4):249–260

Bossaert K, Farnir F, Leclipteux T, Protz M, Lonneux JF, Losson B (2000) Humoral immune response in calves to single-dose, trickle and challenge infections with *Fasciola hepatica*. Vet Parasitol 87:103–123

Carnevale S, Rodriguez MI, Santillan G, Labbe JH, Cabrera MG, Bellegarde EJ, Velasquez JN, Trgovcic JE, Guarnera EA (2001) Immunodiagnosis of human fascioliasis by an enzyme-linked immunosorbent assay (ELISA) and a micro-ELISA. Clin Diagn Lab Immunol 8:174–177

Cornelissen JB, Gaasenbeek CP, Boersma W, Borgsteede FH, van Milligen FJ (1999) Use of a pre-selected epitope of cathepsin-L1 in a highly specific peptide-based immunoassay for the diagnosis of *Fasciola hepatica* infections in cattle. Int J Parasitol 29:685–696

Cornelissen JB, Gaasenbeek CP, Borgsteede FH, Holland WG, Harmsen MM, Boersma WJ (2001) Early immunodiagnosis of fasciolosis in ruminants using recombinant *Fasciola hepatica* cathepsin L-like protease. Int J Parasitol 31:728–737

Demerdash ZA, Diab TM, Aly IR, Mohamed SH, Mahmoud FS, Zoheiry MK, Mansour WA, Attia ME, El-Bassiouny AE (2011) Diagnostic efficacy of monoclonal antibody based sandwich enzyme linked immunosorbent assay (ELISA) for detection of *Fasciola gigantica* excretory/secretory antigens in both serum and stool. Parasit Vectors 4:176

Espino AM, Hillyer GV (2001) Identification of fatty acid molecules in a *Fasciola hepatica* immunoprophylactic fatty acid-binding protein. J Parasitol 87:426–428

Falak R, Sankian M, Noorbakhsh R, Tehrani M, Assarehzadegan MA, Jabbari Azad F, Abojhasani A, Varasteh AR (2013) Identification and characterisation of main allergic proteins in Vitis vinifera vitis. Food Agric Immunol 24:255–268

Farghaly AM, Nada SM, Emam WA, Mattar MA, Mohamed SM, Sharaf EM, Gamal RE (2009) Role of Fast-ELISA and Western blot in diagnosis of human fasciolosis using crude adult worm and excretory/secretory fasciola antigens. PUJ 2:55–65

Gonenc B, Sarimehmetoglu HO, Kara M, Kircali F (2004) Comparison of crude and excretory/secretory antigens for the diagnosis of *Fasciola hepatica* in sheep by Western blotting. Turk J Vet Anim Sci 28:943–949

Hacariz O, Sayers G, Mulcahy G (2011) A preliminary study to understand the effect of *Fasciola hepatica* tegument on naive macrophages and humoral responses in an ovine model. Vet Immunol Immunopathol 139:245–249

Hillyer GV (1999) Immunodiagnosis of human and animal fasciolosis. In: Dalton JP (ed) Fasciolosis. CABI Publishing, Wallingford, pp 435–447

Mas-Coma S, Bargues MD, Valero MA (2005) Fascioliasis and other plant-borne trematode zoonoses. Int J Parasitol 35:1255–1278

McGonigle S, Dalton JP (1995) Isolation of *Fasciola hepatica* haemoglobin. Parasitology 111:209–215

Meshgi B, Eslami A, Hemmatzadeh F (2008) Determination of somatic and excretory-secretory antigens of *Fasciola hepatica* and *Fasciola gigantica* using SDS-PAGE. Iranian J Vet Res 9:77–80

Mezo M, Gonzalez-Warleta M, Ubeira FM (2003) Optimized serodiagnosis of sheep fascioliasis by Fast-D protein liquid chromatography fractionation of *Fasciola hepatica* excretory-secretory antigens. J Parasitol 89:843–849

Mohamed MM, Al-Sherbiny MM, Sharaf AA, Elmamlouk TH (2004) Immunological identification of *Fasciola hepatica* antigens containing major human T-cell and B-cell epitopes. J Egypt Soc Parasitol 34:751–766

Morales A, Espino AM (2012) Evaluation and characterization of *Fasciola hepatica* tegument protein extract for serodiagnosis of human fascioliasis. Clin Vaccine Immunol 19:1870–1878

Rivera-Marrero CA, Santiago N, Hillyer GV (1988) Evaluation of immunodiagnostic antigens in the excretory-secretory products of Fasciola hepatica. J Parasitol 74:646–652

Robinson MW, Menon R, Donnelly SM, Dalton JP, Ranganathan S (2009) An integrated transcriptomics and proteomics analysis of the secretome of the helminth pathogen *Fasciola hepatica*: proteins associated with invasion and infection of the mammalian host. Mol Cell Proteomics 8:1891–1907

Rokni MB, Massoud J, O'Neill SM, Parkinson M, Dalton JP (2002) Diagnosis of human fasciolosis in the Gilan province of Northern Iran: application of cathepsin L-ELISA. Diagn Microbiol Infect Dis 44:175–179

Rokni MB, Baghernejhad A, Mohebali M, Kia EB (2004) Enzyme linked immunotransfer blot analysis of somatic and excretory-secretory antigens of *Fasciola hepatica* in diagnosis of human fasciolosis. Iran J Publ Health 33:8–13

Rokni MB, Mirhendi H, Behnia M, Fasihi Harandi M, Jalalizand N (2010) Molecular characterization of *Fasciola hepatica* isolates by RAPD-PCR and ribosomal ITS1 sequencing. Iranian Red Crescent Med J 12:27–32

Sampaio-Silva ML, Da Costa JM, Da Costa AM, Pires MA, Lopes SA, Castro AM, Monjour L (1996) Antigenic components of excretory-secretory products of adult *Fasciola hepatica* recognized in human infections. Am J Trop Med Hyg 54:146–148

Sarkari B, Ghobakhloo N, Moshfea A, Eilami O (2012) Seroprevalence of human fasciolosis in a new-emerging focus of fasciolosis in Yasuj District, Southwest of Iran. Iran J Parasitol 7:15–20

Spithill TW, Smooker PM, Sexton JL, Bozas E, Morrison CA, Creaney J, Parsons JCJP (1999) Development of vaccines against *Fasciola hepatica*. In: Dalton JP (ed) Fasciolosis. CABI Publishing, Wallingford, pp 377–401

Upadhyay AK, Kumar M (2002) SDS-PAGE analysis of *Fasciola gigantica* antigen. J Immunol Immunopathol 4:91–92

Valero MA, Periago MV, Perez-Crespo I, Rodriguez E, Perteguer MJ, Garate T, Gonzalez-Barbera EM, Mas-Coma S (2012) Assessing the validity of an ELISA test for the serological diagnosis of human fascioliasis in different epidemiological situations. Trop Med Int Health 17:630–636

Yamano K, Goto A, Nakamura-Uchiyama F, Nawa Y, Hada N, Takeda T (2009) Galbeta1-6Gal, antigenic epitope which accounts for serological cross-reaction in diagnosis of Echinococcus multilocularis infection. Parasite Immunol 31:481–487